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Identification of Bacterial Muramyl Dipeptide as Activator of the NALP3/Cryopyrin Inflammasome

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Summary

Activation of caspase-1 and subsequent processing and secretion of the pro-inflammatory cytokine IL-1β is triggered upon assembly of the inflammasome complex [1]. It is generally believed that bacterial lipopolysaccharides (LPS) are activators of the inflammasome through stimulation of Toll-like receptor 4 (TLR4) [2]. Like TLRs, NALP3/Cryopyrin, which is a key component of the inflammasome [3], contains Leucine-Rich-Repeats (LRRs). LRRs are frequently used to sense bacterial components [1, 4, 5], thus raising the possibility that bacteria directly activate the inflammasome. Here, we show that bacterial peptidoglycans (PGN), but surprisingly not LPS, induce NALP3-mediated activation of caspase-1 and maturation of proIL-1β. Activation is independent of TLRs because the PGN degradation product muramyl dipeptide (MDP), which is not sensed by TLRs, is the minimal-activating structure. Macrophages from a patient with Muckle-Wells syndrome, an autoinflammatory disease associated with mutations in the NALP3/Cryopyrin gene, show increased IL-1β secretion in the presence of MDP. The activation of the NALP3-inflammasome by MDP may be the basis of the potent adjuvant activity of MDP.

Results and Discussion

The pivotal molecules of the inflammasome are the NALP proteins, a family of proteins encompassing 14 members [6, 7]. NALPs contain a Pyrin domain, a NOD/NACHT oligomerization domain, and LRRs. NALP1, NALP2, NALP3, NALP6, and NALP12 bind and activate caspase-1 via the adaptor protein ASC [3, 5, 6, 8]. Albeit less efficient than the NALPs, a NALP-related protein called Ipaf has also been shown to activate caspase-1 [5, 9–11]. LRRs found in both the NALPs and Ipaf are interaction domains that are frequently used in so-called pattern recognition receptors (PRR) to detect key molecular signatures of invading pathogens, i.e., pathogen-associated molecular patterns (PAMPS), thereby triggering the innate immune system [12]. Examples of PRR are TLRs, which detect bacterial or viral products such as LPS (TLR4) [13]. It was therefore possible that the LRRs of some of the NALP proteins would directly sense bacterial products, leading to the activation of caspase-1 independent of TLRs.

In order to test this hypothesis, we added crude preparations of LPS (LPSc) or ATP, which is another well-known stimulus, to the monocytic cell line THP-1 (Figures 1A and 1B). Intracellular processing of proIL-1β to the active p17 fragment occurred in cells within 1 hr, followed by the release of the active cytokine after 2–3 hr. To our surprise, however, highly purified *E. coli* LPS (LPSp) did not stimulate IL-1β processing and secretion (Figures 1A and 1B). The trivial explanation that LPSp was inactive was ruled out because addition of LPSp to mouse embryonic fibroblasts resulted in the robust phosphorylation of the inhibitor of NF-κB, I-κB, and phosphorylation (activation) of extracellular signal-regulated kinases (ERK) in a time-dependent manner (Figure 1C). Therefore, we suspected that the active component triggering IL-1β maturation was not LPS but a contaminant thereof. The crude LPS mixture was therefore separated by gel-filtration chromatography. Subsequent analysis of the fractions revealed a broad peak of IL-1β-processing activity, confirming the heterogeneity of the preparation (Figure 2A, lower panel). A significant activity eluted in low molecular weight fractions in which LPS was unlikely to be present (LPS micelles elute in the void volume fractions). This activity profile was strikingly reminiscent of that shown for the identification of the bacterial component responsible for the activation of NOD2 [14]. NOD2 and its structural relative NOD1 are intracellular PRR and have high structural similarity to the NALPs, but their stimulation leads to the activation of the NF-κB pathway rather than to caspase-1 activation [8]. Both NOD2 and NOD1 sense the presence of bacterial PGN [14–16].

Considering the structural similarity of NODs and NALPs and the similarity of the gel-filtration activity profiles, we hypothesized that PGN products may also act as a PAMP for the NALPs. The crude LPS preparation was therefore treated with mutanolysin, an enzyme that digests PGN, but not LPS, yielding muramyl-peptides composed of N-acetylglucosamine (GlcNAc) and N-acetyl muramic acid (MurNAc) linked to small peptides (Figure 2B). After gel-filtration chromatography, the IL-1β-processing activity had indeed shifted and eluted predominantly in two peaks with small molecular masses (Figure 2B). These results are consistent with the notion that the IL-1β-activating substance is not LPS but PGN and muropeptides thereof. Indeed, exposure of THP-1 cells to PGN alone efficiently induced secretion of IL-1β into the supernatant (Figure 2C), while other PAMPs of TLRs such as Poly-IC (TLR3), CpG (TLR9), Pam_3Cys (TLR2), and Zymosan (TLR2) had no effect.

The absence of proIL-1β-processing activity of Pam_3Cys and Zymosan made it unlikely that PGN products activate caspase-1 and IL-1β by binding to TLR2. Still, in order to refute this possibility and to avoid any contamination by other bacterial substances found in crude polymeric PGN preparations, we used a synthetic monomeric derivative of PGN, i.e., the muramyl-dipep-

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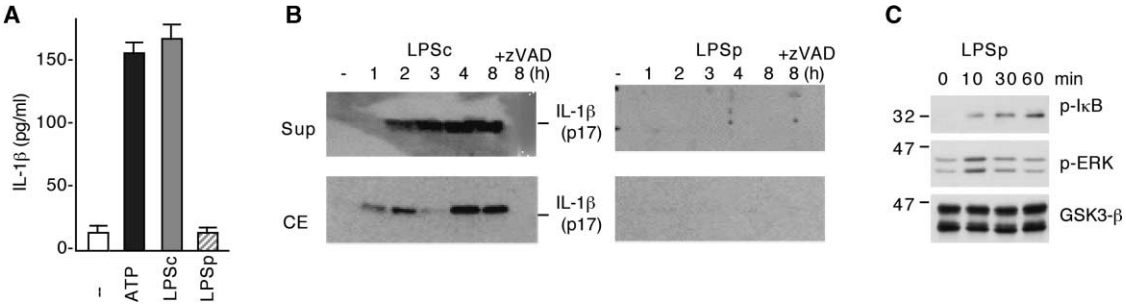

Figure 1. LPS Does Not Activate proIL-1β

(A) Concentrations of active IL-1β in supernatants of THP-1 cells stimulated for 6 hr with ATP (5 mM), crude LPS (LPSc, 10 μg/ml), and a preparation of ultrapure LPS (LPSp) (10 μg/ml) were determined by ELISA. Values represent mean of normalized data ± SD of triplicate cultures.

(B) Western blotting and an antibody (D116) that specifically recognizes processed IL-1β (p17) was used to monitor maturation of proIL-1β in cell extracts (CE) and supernatants (Sup) of THP-1 cells stimulated with either crude (LPSc) or pure preparations (LPSp) of LPS. Where shown, cells were preincubated with zVAD-fmk.

(C) Mouse embryonic fibroblast (MEFs) were stimulated with LPSp (1 μg/ml) as indicated and analyzed by Western blotting for the activation of I-κB and ERK using phospho-specific antibodies. As a loading control, GSK3-β was used.

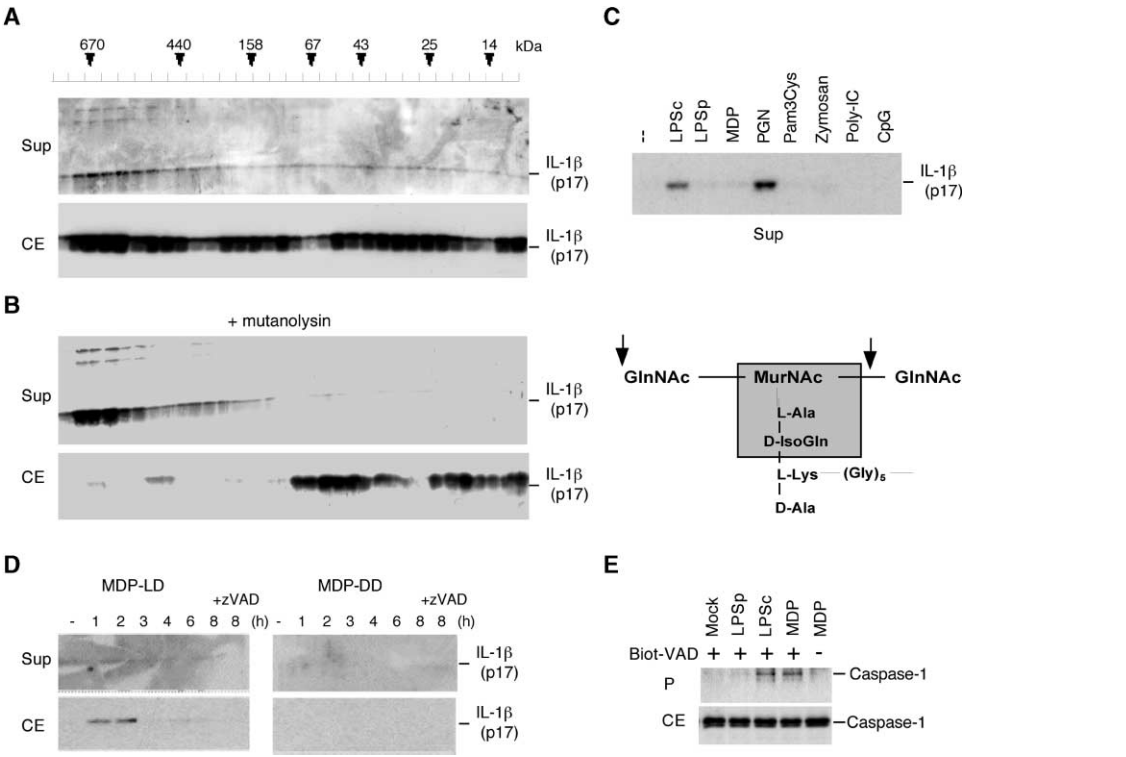

Figure 2. PGN and Muramyl-dipeptides induce IL-1β maturation

(A) Crude LPS was fractionated by gel-filtration chromatography. THP-1 cells were then treated for 3 hr with the column fractions. Active IL-1β was measured in cell extracts (CE) or supernatants (Sup) by Western blotting as described in Figure 1.

(B) As (A), but the crude LPS was digested with mutanolysin prior to gel-filtration chromatography. Mutanolysin cleaves PGN between MurNAc and GlnNAc as indicated in the schematic representation. The box indicates the structure of Muramyl dipeptide (MDP).

(C) THP-1 cells were treated with crude LPS (10 μg/ml), LPSp (10 μg/ml), TLR9-activating CpG (200 nM), TLR3-activating Poly-IC (50 μg/ml), TLR2-activating Pam_3Cys (10 μg/ml), Zymosan (10 μg/ml), and PGN (1 μg/ml); cleaved IL-1β was subsequently determined in supernatants as described above.

(D) The IL-1β-processing activity of synthetic MDP-LD and MDP-DD was determined at various time points by monitoring the appearance of cleaved IL-1β with the D116 antibody. Where indicated, the pan-caspase-inhibitor zVAD-fmk was added to block caspase-1.

(E) Activation of caspase-1 by MDP. Cells were incubated with biotinylated VAD-fmk (30 μM) and subsequently stimulated with MDP or LPS peparations for 2 hr. Active caspase-1 was then precipitated (P) using streptavidin beads. Precipitates and cell extracts (CE) were subsequently analyzed for the presence of caspase-1 by Western blotting.

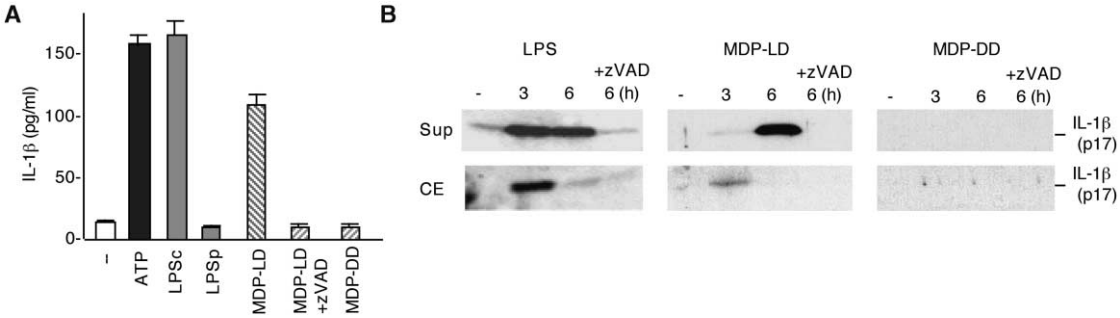

Figure 3. Synthetic Muramyl Dipeptide Induces IL-1β Maturation and Release in Primary Human Macrophages

Primary human macrophages were exposed to ATP (5mM), crude LPS (LPSc, 10 μg/ml), pure LPS (LPSp, 10 μg/ml), MDP-LD (3μg/ml), and MDP-DD (3 μg/ml) in the presence or absence of zVAD-fmk for 6 hr or as indicated. IL-1β release and processing were subsequently measured by ELISA (A) and Western blotting using the D116 antibody specifically detecting the active fragment (p17) of IL-1β (B).

tide MurNAc-L-Ala-D-isoGln (MDP-LD) to stimulate THP-1 cells. Synthetic MDP-LD and pure PGN do not activate TLR2 [14, 17, 18]. Addition of MDP-LD to THP-1 cells caused rapid processing of intracellular proIL-1β, detectable after 1 to 2 hr (Figure 2D), whereas the stereoisomer MurNAc-D-Ala-D-isoGln (MDP-DD) was inactive, indicating specificity and independence of TLR2. Because proIL-1β maturation is dependent on caspase-1 activation, we also studied the activation of this proinflammatory caspase. Caspase-1 activation is difficult to detect in cell extracts because active caspase-1 is rapidly secreted upon inflammasome assembly [4, 5]. Therefore, we trapped intracellular active caspase-1 by using the biotinylated pan-caspase inhibitor VAD-fmk, which binds to active caspases. Active caspase-1 in its noncleaved form (processing is not required for initiator caspases [19]) was detectable in cell extracts in the presence of MDP and contaminated LPSc, but not LPSp (Figure 2E).

Although synthetic MDP-LD induced processing of proIL-1β in the cytoplasm, subsequent release of the active cytokine was very inefficient in THP-1 cells (Figure 2D). Release of IL-1β was only observed upon treatment of cells with crude preparations of LPS or PGN (Figure 2C). This finding is in agreement with the activity profile of fractionated LPSc (Figure 2A), where only high-molecular fractions (most likely corresponding to polymeric PGN) induced efficient IL-1β release. Thus, at least in THP-1 cells, intracellular processing of proIL-1β and subsequent release appear to be two separate events. Interestingly, these two distinct steps were not observed in primary cells. When MDP was added to purified human monocytes, active IL-1β was detectable in the cytoplasm as well as in the supernatant (Figures 3A and 3B). Pretreatment of cells with zVAD-fmk inhibited both processing and release of IL-1β.

Mutations in the NOD/NACHT domain of NALP3/Cryopyrin have been genetically associated with a broad phenotypic spectrum of dominantly inherited autoinflammatory disorders, including Muckle-Wells syndrome (MWS), familial cold urticaria (FCU), and chronic neurologic cutaneous and articular syndrome (CINCA) [20–23]. All three disorders are characterized by recurrent inflammatory crises that involve fever, rash, and arthritis [24]. The almost-20 distinct mutations found in the NALP3 gene localize to the NOD/NACHT domain and have been shown or predicted to lead to some constitutive activation of IL-1β and to a hypersensitivity to exogenous stimuli [3, 25, 26]. Macrophages from a patient with MWS (R260W mutation) were therefore exposed to MDP and the amount of processed IL-1β measured in the supernatant (Figure 4). When compared to control macrophages and measured 2 and 6 hr poststimulation, the IL-1β concentration was approximately 2-fold and 6-fold higher in supernatants of cells from the MWS patient (Figures 4A and 4B). No difference was observed with respect to TNF secretion (Figure 4C), indicating that MDP-mediated TNF secretion is not affected by NALP3 mutations.

The above results strongly suggest that IL-1β processing in response to MDP was triggered through activation of NALPs. However, we could not formally exclude the involvement of the other NOD-like proteins implicated in caspase-1 activation, such as Ipaf. To obtain direct evidence that MDP can activate NALPs, we transfected 293T cells with all components required for the NALP3 inflammasome assembly, i.e., NALP3, ASC, caspase-1, and proIL-1β. Figure 5A shows that active IL-1β was detectable in the cytoplasm of transfected cells in the presence but not in the absence of MDP. Processing was dependent on the presence of ASC, NALP3, and caspase-1, indicating that MDP activated the reconstituted inflammasome and not endogenous proteins present in nontransfected cells. As previously shown [14], the same concentrations of MDP also led to the activation of NOD2 (Figure 5B). Again, only the addition of crude but not pure LPS preparation triggered inflammasome activation (Figures 5A and 5C).

In order to avoid spontaneous inflammasome activation, the LRRs of NALP3 interact with the NOD/NACHT domain, thereby inhibiting NALP3 activation, which is independent of the presence of an activating ligand [3]. NALP3 exists in at least four isoforms, the shortest of which (NALP3s) is devoid of LRRs [21, 27]. Overexpression of NALP3s in 293T cells containing the other inflammasome components resulted in the spontaneous activation of IL-1β, which was not further enhanced in the presence of MDP (Figure 5C), indicating that MDP-mediated inflammasome activation is dependent on the LRRs of NALP3.

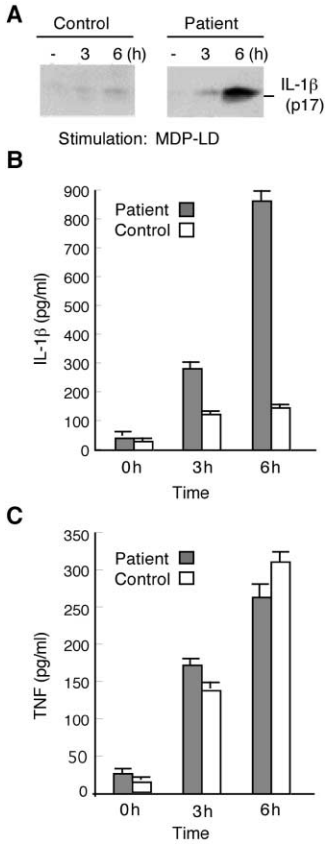

Figure 4. Increased IL-1β Processing in Macrophages Isolated from a Muckle-Wells Patient in the Presence of MDP

Macrophages from a Muckle-Wells patient carrying the R260W mutation or a nonaffected donor were left unstimulated or stimulated with MDP for 3 and 6 hr. IL-1β present in the supernatant was measured by Western blot analysis (A) or by ELISA (B) as described in Figure 3. (C) Concentrations of TNF in the supernatant of MDP-stimulated macrophages were determined by ELISA.

The data presented here demonstrate that MDP is the minimal structure that allows activation of the NALP3 inflammasome. Recently, MDP was also identified as the minimal bacterial degradation product able to activate NOD2 [14, 28]. MDP is present in PGN of most gram-positive and gram-negative bacteria. Therefore, NALP3 can detect a diversity of bacteria, but how MDP activates NALP3 (and NOD2) is currently unknown. Macrophages contain intracellular hydrolases that digest PGN of intracellular and phagocytosed bacteria, generating MDP, amongst other products. However, activation of NALP3 ultimately depends on the capacity of PGN products to translocate to the cytoplasm. Once cytosolic, MDP could then interact either directly with the LRRs of NALP3 or indirectly by means of a linker protein.

The NALP family comprises 14 members. In addition to NALP3, we found that NALP1 and NALP2 also sense the presence of MDP (unpublished data). NOD1, although structurally closely related to NOD2, does not recognize MDP but γ-D-iso-glutamyl diaminopimelic acid. Thus, it is likely that not all NALPs sense MDP, considering also that some of the NALPs are not expressed in immune cells.

We found that pure LPS failed to induce IL-1β processing and secretion. This was surprising as LPS is widely used to stimulate IL-1β processing, demonstrating that most commercially available LPS preparations are not pure and must contain substantial quantities of PGN and muramyl peptides. Interest in muramyl peptides was already sparked 30 years ago by the identification of MDP as the active component of Freund's complete adjuvant [29]. However, its mechanism of action remained elusive. IL-1β is a major mediator of inflammation. For this reason, IL-1β activity is tightly controlled. IL-1β mRNA is absent from cells until stimulated by signals activating NF-κB. Even then, the translated protein is inactive and needs proteolytic processing. MDP, as an activator of both NF-κB via NOD2 and caspase-1 via NALPs, fulfills all the criteria required for the initiation of an optimal innate immune response.

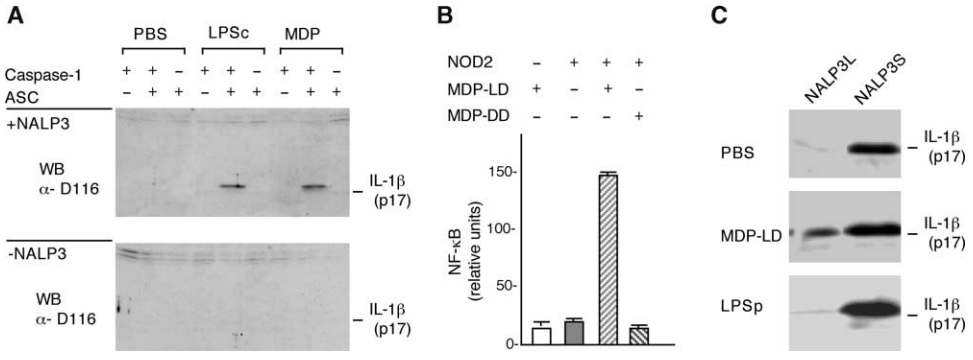

Figure 5. Activation of NALP3 by Synthetic MDP

(A) 293T cells were transfected with low amounts of expression plasmids encoding ASC, caspase-1, and proIL-1β (10 ng of each plasmid) with (upper panel) or without (lower panel) a plasmid encoding NALP3 (10 ng). During transfection, the cells were exposed to crude LPS (LPSc) (10 μg/ml) or synthetic MDP (2 μg/ml). 24 hr posttransfection, the presence of the active cleaved form of IL-1β in cell extracts was determined by Western blotting.

(B) MDP-mediated stimulation of NOD2. 293T cells were transfected with NOD2 (60 ng) and a NF-κB reporter gene in the presence and absence of MDP (2 μg/ml). The activity of the reporter gene was assayed 26 hr posttransfection.

(C) Processing of IL-1β in response to MDP and LPSp was compared in 293T cells that express the long form of NALP3 with 293T cells that express the short form of NALP3 (NALP3s), which lacks the LRR.

Macrophages of a patient with MWS were found to be overresponsive to MDP, resulting in secretion of substantial amounts of active IL-1β. Recurrent fever attacks are one of the symptoms of patients with autoinflammatory diseases [24]. Because injection of MDP causes fever [30], and IL-1β is the most potent endogenous pyrogen currently known [31], it is possible that the patients' recurrent fever attacks are directly linked to increased MDP levels. Antagonists of muramyl peptides may therefore be of clinical value for the treatment of autoinflammatory diseases [32].

Supplemental Data

Supplemental Data including Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/14/21/1929/DC1/.

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